



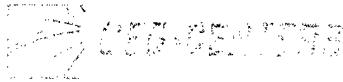
EDGEWOOD

CHEMICAL BIOLOGICAL CENTER

U.S. ARMY SOLDIER AND BIOLOGICAL CHEMICAL COMMAND

ECBC-TR-243

**PROCESS DEVELOPMENT FOR THE PRODUCTION
OF A MONOCLONAL ANTIBODY
AGAINST *FRANCISELLA TULARENSIS***



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PREFACE

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PROCESS DEVELOPMENT FOR THE PRODUCTION OF A MONOCLONAL ANTIBODY AGAINST *FRANCISELLA TULARENSIS*

1. INTRODUCTION

Monoclonal antibodies (mAbs) are the component in biosensors that specifically bind biological threat agents, providing a capability essential to fulfilling the biological defense mission of the Department of Defense (DoD). The use of mAbs specific for biological warfare (BW) agents in fielded assays requires the maintenance of a high-quality and economical supply of these critical reagents. Monoclonal antibodies are produced by hybridoma cells, fusions between mouse antibody-producing cells and mouse cancer cells, which have no finite lifespan and can be grown in culture indefinitely. Hybridomas have traditionally been grown in the peritoneum of live mice, where they produce antibodies in ascites tumor fluid. This method, essentially the use of mice as living bioreactors, can obtain high concentrations of antibody. However, occasionally hybridomas produce abdominal tumors rather than ascites fluid, necessitating the growth of the cell line *in vitro* to obtain antibodies. In addition, there is a growing ethical concern about the humaneness for producing mAb using this method. An increased emphasis on development of better *in vitro* methods has generated several new techniques that boost the yield of mAbs without the use of live animal hosts.^{1,2}

A variety of cell culture vessels have been devised for the small-scale production of mAb (milligrams to a few grams). Pilot studies were conducted comparing three *in vitro* cell culture methods to determine which gave the highest yield of a mAb that binds the BW agent, *Francisella tularensis*, in the shortest amount of time.³ The FT-03, a hybridoma cell line producing an anti-*F. tularensis* antibody was grown in 1-L flask-type bioreactors (Integra CELLline CL1000 flasks), a hollow fiber bioreactor, and 2-L gas permeable bags. Two-liter bags were subsequently chosen for the production of approximately 10 g of antibody.

Monoclonal antibodies, after being produced and secreted by hybridoma cells growing *in vitro*, must be purified from the spent culture medium. Several techniques are available, which include mixed mode chromatography (e.g., hydroxy-apatite), physiochemical chromatography mechanisms (e.g., ion exchange, gel filtration, or hydrophobic), and non-chromatographic methods (e.g., ammonium sulfate precipitation). Affinity column chromatography, using a resin coated with Protein-A, is a highly effective and efficient method, and this method was employed in this study. Protein-A is a cell wall component of *Staphylococcus aureas*, which specifically binds the Fc regions of various mAb subclasses from several species. A recombinant form of Protein-A, which lacks the cell wall associated region and has antibody binding activity

*Park, J. T.; Cork, S.; Coliano, T.; Cao, C.; Menking, D.; O'Connell, K.; and Valdes, J. J.; Research and Technology Directorate, U.S. Army Edgewood Chemical Biological Center, July 2002, unpublished data.

indistinguishable from the original version, is now commonly used.² Widely used for the purification of mAbs from spent culture media, especially those containing fetal bovine serum, Protein-A chromatography precludes co-purification of contaminants that may be present in the cell cultures.³

In this study, we describe the process development and production of a mAb against *Francisella tularensis* from hybridoma cultures grown in flexible, gas permeable, tissue culture bags. After the growth of anti-*F. tularensis* antibody-secreting hybridoma cells, the resulting antibodies were purified by affinity chromatography purification, using a recombinant Protein-A column and formulated in Tris-buffer (pH 8.0). Samples collected during cell culture, purification, and formulation were analyzed by several techniques for the process monitoring/optimization and the determination of yield. The purity and activity of batches of the final mAb product were determined by high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assays (ELISA) analysis, respectively.

2. MATERIALS AND METHODS

2.1 Production of FT03 mAb.

2.1.1 Materials.

Becton Dickinson (BD) Cell mAb Medium Quantum Yield (BD Cell Media) was purchased from BD Bioscience (Sparks, MD) and fetal bovine serum (FBS) was purchased from Life Technologies (Grand Island, NY). The T-25, T-75, and T-150 flasks were purchased from Becton Dickinson and Company (Franklin Lakes, NJ). Both 300 mL (catalog #163212) and 2-L (catalog #165412) gas permeable bags were purchased from TC Technologies (Minneapolis, MN). Milligard Opticap prefilters were purchased from Millipore Corporation (Bedford, MA). Protein-A sepharose fast flow and Sephadex G-25 desalting resins were purchased from Amersham Biosciences (Piscataway, NJ). The XK26 and XK50 empty columns were purchased from Amersham Biosciences. Trizma base (T-6066), Trizma hydrochloride (T-5941), sodium chloride (S-9888), and sodium azide (S-8032) were purchased from Sigma Chemical Company (St. Louis, MO). Sodium citrate dihydrate (catalog #3649) and citric acid monohydrate (catalog #0119) were purchased from J. T. Baker (Phillipsburg, NJ). All other chemicals used are at least American Chemical Society (ACS) grade.

2.1.2 Hybridoma Cell Culture.

A process diagram for the production of a mAb against *F. tularensis* is shown in Figure 1. A hybridoma cell line that produces anti-*F. tularensis* antibodies was selected from our in-house hybridoma collection. The BD Cell media were supplemented with 17.6% fetal calf serum, 100 units/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, and 2 mM L-glutamine. This medium is referred to as "BD Cell Complete" media and was used throughout the cell culture process.

One milliliter of the cell line was thawed, washed, and resuspended in the BD Cell Complete media and transferred into a T-25 flask (25-cm² area). As these cells multiplied, they were gradually expanded to T-75 flasks (75-cm² area) and finally to T-150 flasks (150-cm² area). Generally, one to two T-150 flasks can be used to inoculate one 300 mL gas permeable bag. One 300 mL bag can be used to inoculate two 2-L gas permeable bags. These 2-L bags were incubated at 37 °C in a humidified 5% (by volume) CO₂ atmosphere for 3 - 4 weeks in a Steri-Cult 200 CO₂ incubator (Forma Scientific, Marietta, OH). At the end of incubation, media and cells were harvested by centrifugation using an Avanti Centrifuge (Beckman Coulter, Fullerton, CA). The cell supernatant was retained for purification.

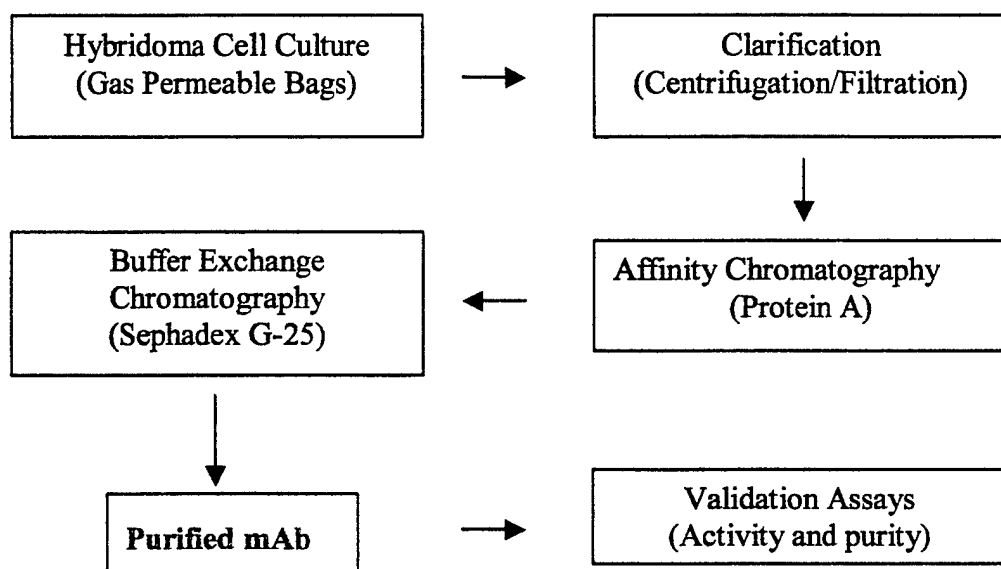


Figure 1. Schematic Process Diagram of the Production, Purification, and Testing of Monoclonal Antibody

2.1.3 Purification of mAb.

Monoclonal antibodies were purified by a simple, single column process using a recombinant Protein-A affinity resin. The cell media harvested from five 2-L gas permeable bags was pre-filtered through a 0.5/0.2 µm disposable Opticap filter. The filtered cell culture media at approximately pH 7.4 was loaded onto the Protein-A column (~ 60 mL bed volume) and eluted with sodium citrate buffer, pH 3.0. To preserve antigen-binding activity, fractions of the eluent were collected in tubes containing 1 M Tris buffer (pH 9.0). Quick neutralization of the acidity of the eluent was important, as maintaining the antibody at low pH is believed to reduce its activity over time. Antibody-containing fractions were pooled, and a buffer exchange into the final product buffer, [Tris-buffered saline (TBS), pH 8.0 including 0.05% sodium azide] was accomplished using a Sephadex G-25 desalting column (~ 400 mL bed volume). Tris-based buffers maintain the solubility of this particular mAb product and discourage

recipitation during storage. Finally, antibody solutions were sterilized by passage through a 0.22 µm filter.

2.2 Analytical Methods.

2.2.1 Materials.

Protein-A Sepharose Fast Flow resin was purchased from Amersham Pharmacia Biotech (Piscataway, NJ) and empty HPLC columns (4.6 mm ID x 50 mm L) were obtained from PE Biosystem (Framingham, MA). Analytical gel permeation chromatography (GPC) TSK SWXL guard column (part #08543) and TSK G3000SWXL (part #08541) were purchased from Tosoh Biosep LLC (Montgomeryville, PA). Mouse IgG and Bovine serum albumin (BSA) were obtained from Sigma Chemical Company (St. Louis, MO). Sodium phosphate monobasic dihydrate and sodium phosphate dibasic heptahydrate were purchased from J. T. Baker (Phillipsburg, NJ). All other chemicals used are at least ACS grade.

2.2.2 Trypan Blue Method for the Cell Count and Viability Measurement.

Fifty microliters of cell suspension was removed from the total volume of cell chamber contents and diluted with 450 µL of complete medium. Fifty microliters of this suspension was diluted with 50 µL trypan blue dyes. This was applied to a hemacytometer and four fields of 16 squares were counted. Dead cells were blue and viable cells were white/clear. Total cell number per milliliter of suspension from bioreactor = $(\text{dead} + \text{live} / 4) \times 20 \times 1\text{E}4$. Viable cell number per milliliter = $(\text{live cells} / 4) \times 20 \times 1\text{E}4$. Percent viability = $(\text{live cells} / \text{total cells}) \times 100$.

2.2.3 Analytical Protein-A HPLC Method for the mAb Quantification.⁴

The HPLC system used for the assay consists of a HPLC pump (Beckman Coulter, System Gold Model 118) (Beckman Coulter, Fullerton, CA), an autosampler (Beckman System Gold Model 508), and an UV detector (Beckman Coulter, System Gold Model 166). Chromatographic data were acquired and analyzed using 32-Karat software (Beckman Coulter). To pack a Protein-A column, an empty HPLC column (0.8 mL) was connected to a Poros-SelfPack Packing Device (PE Biosystem), and the slurry of Protein-A resin was poured all at once into the assembly. The assembly with Protein-A resin slurry is then connected to a HPLC system, and the loading PBS Buffer was pumped into the top of the packing device at a 1.0 mL/min flow rate to pack the column. After packing, the packing device was removed, and the column was attached to the column end fitting. For each analysis, samples were pretreated by filtering with a 0.2-µm filter or micro-centrifuging before injection. After injection, the phosphate buffered saline (PBS) buffer at pH 7.2 (20 mM sodium phosphate/150 mM sodium chloride) was used a loading/washing buffer at the rate of 0.5 mL/min for 10 min. A target mAb was eluted with the 50 mM citrate buffer at pH 3 using a linear gradient at a rate of 0.5 mL/min for 10 min. The column was then washed with the citrate buffer (pH 3) for 5 min at 0.5 mL/min after the elution of target mAbs.

2.2.4 Analytical Chromatography Methods for Assessing mAb Purity.

A HPLC system similar to that mentioned in Section 2.2.3 was used for the measurement of mAb purity. Samples were injected through a single GPC TSK G3000SWXL column. The purity of mAb was measured by 32-Karat software.

2.2.5 ELISA Assay for Assessing mAb Antigen Binding Activity.

2.2.5.1 Coating Plates with Antigen.

The *F. tularensis* antigen (Dugway Proving Ground, UT) was diluted to 5 µg/mL in phosphate buffered saline (PBS; 0.01 M phosphate, 0.15 M NaCl, pH7.4; Sigma Company, St. Louis, MO) and placed in each well of rows A, C, E, and G in a 96-well microtiter plate. Negative control antigen (bovine serum albumin; Sigma Company) was diluted to 5 µg/mL in PBS and placed in each well of rows B, D, F, and H of the same plate. The *F. tularensis* antigen concentration conformed to the U.S. Army Edgewood Chemical Biological Center-sanctioned standing operating procedure for antibody quality conformity testing. Microtiter plates (U-bottomed; Nunc MAXISorp or Dynex Immulon II) were sealed with transparent plate sealing membranes and incubated overnight at 4 °C. Following overnight incubation, plates were washed twice using ELISA wash buffer (PBS, 0.1% Tween 20, 0.0001% thimerosal) in a ScanWasher Model 300B (Skatron).

2.2.5.2 Binding Primary Antibodies.

Reference ("gold standard") antibody and the antibody to be tested were serially diluted in ELISA dilution buffer (ELISA wash buffer plus 50 g dry skim milk/L). First, 100 µL dilution buffer was added to all wells in each assay plate. Reference and test antibodies were diluted to a concentration of 40 µg/mL in ELISA dilution buffer, and 100 µL (4 µg) of each were added to the plate as follows:

- The reference antibody was added to wells A1 and B1.
- The antibody to be tested was added to wells E1 and F1.

The final concentration of each antibody in A1, B1, E1, and F1 was therefore 20 µg/mL (4 µg in a total volume of 200 µL). Antibodies were serially diluted as shown in Figure 2, by transferring 100 µL from wells in the first column to the second column, mixing by pipetting, then transferring 100 µL to wells in the third column and so on. Plates were incubated in a Labsystems iEM incubator/shaker type 1410 (37 °C, 400 rpm, 1 hr) followed by two washes in the SkanWasher as before.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|-----------|-----------|-----------|-----------|------------|-----------|------------|------------|----------|----------|------------|-----------|
| A | 20 µg/mL | 10 µg/mL | 5 µg/mL | 2.5 µg/mL | 1.25 µg/mL | 625 ng/mL | 313 ng/mL | 156 ng/mL | 78 ng/mL | 39 ng/mL | 19.5 ng/mL | 9.8 ng/mL |
| B | 20 µg/mL | 10 µg/mL | 5 µg/mL | 2.5 µg/mL | 1.25 µg/mL | 625 ng/mL | 313 ng/mL | 156 ng/mL | 78 ng/mL | 39 ng/mL | 19.5 ng/mL | 9.8 ng/mL |
| C | 4.9 ng/mL | 2.4 ng/mL | 1.2 ng/mL | 610 pg/mL | 305 pg/mL | 153 pg/mL | 76.2 pg/mL | 38.1 pg/mL | 19 pg/mL | Blank | Blank | Blank |
| D | 4.9 ng/mL | 2.4 ng/mL | 1.2 ng/mL | 610 pg/mL | 305 pg/mL | 153 pg/mL | 76.2 pg/mL | 38.1 pg/mL | 19 pg/mL | Blank | Blank | Blank |
| E | 20 µg/mL | 10 µg/mL | 5 µg/mL | 2.5 µg/mL | 1.25 µg/mL | 625 ng/mL | 313 ng/mL | 156 ng/mL | 78 ng/mL | 39 ng/mL | 19.5 ng/mL | 9.8 ng/mL |
| F | 20 µg/mL | 10 µg/mL | 5 µg/mL | 2.5 µg/mL | 1.25 µg/mL | 625 ng/mL | 313 ng/mL | 156 ng/mL | 78 ng/mL | 39 ng/mL | 19.5 ng/mL | 9.8 ng/mL |
| G | 4.9 ng/mL | 2.4 ng/mL | 1.2 ng/mL | 610 pg/mL | 305 pg/mL | 153 pg/mL | 76.2 pg/mL | 38.1 pg/mL | 19 pg/mL | Blank | Blank | Blank |
| H | 4.9 ng/mL | 2.4 ng/mL | 1.2 ng/mL | 610 pg/mL | 305 pg/mL | 153 pg/mL | 76.2 pg/mL | 38.1 pg/mL | 19 pg/mL | Blank | Blank | Blank |

Figure 2. Diagram of 96-Well Microplate for Direct ELISA Assay. Wells in rows A, C, E, and G were coated with *F. tularensis* antigen, while wells in rows B, D, F, and H were coated with the negative control antigen (BSA). Rows A, B, C, and D contain the "gold standard" or reference antibody to which the test antibody is being compared. Rows E, F, G, and H contain the test antibody to be validated. The blanks serve as a conjugate control for the non-specific interaction of the coated antigen with the HRP conjugated antibody.

2.2.5.3 Binding Secondary Antibody-HRP Conjugate.

Anti-species IgG (H+L)-HRP conjugated antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was diluted to 200 ng/mL in ELISA dilution buffer, and 100 µL added to each well. Plates were incubated and washed as above. The ABTS substrate solution (Kirkegaard and Perry Laboratories) was prepared fresh for each experiment, and 100 µL was added to each well. Plates were incubated for 5 min at 37 °C in the microplate incubator. Following incubation, plates were read at a wavelength of 405 nm in a MRX microplate reader (Dynex Technologies, Chantilly, VA), programmed to subtract the optical density reading of the BSA-coated rows (negative controls) from the corresponding *F. tularensis*-coated rows (rows containing the same primary antibody).

3. RESULTS

3.1 Hybridoma Cell Culture.

Cultivation of hybridoma cells in flexible, gas permeable disposal bags worked well for antibody production. When compared to other small-scale cell culture techniques, as Integra CL1000 and hollow fiber reactor, a 2-L gas permeable bag produced more total antibody as shown in our previous study. Therefore, gas

*Park, J. T.; Cork, S.; Coliano, T.; Cao, C.; Menking, D.; O'Connell, K.; and Valdes, J. J.; Research and Technology Directorate, U.S. Army Edgewood Chemical Biological Center, July 2002, unpublished data.

permeable bag cell culture technique was chosen for mAb production. Cell density and viability in the media were determined by Trypan Blue method and the data are shown in Figure 3. Total hybridoma cell densities increased progressively, and maximum cell numbers in the bag reached and peaked at approximately 6.4×10^6 cells/mL within 2 weeks of seeding the cells in gas-permeable bag. Live cell concentrations increased within the first week of the culture and diminished slowly to approximately 5.8×10^5 cells/mL.

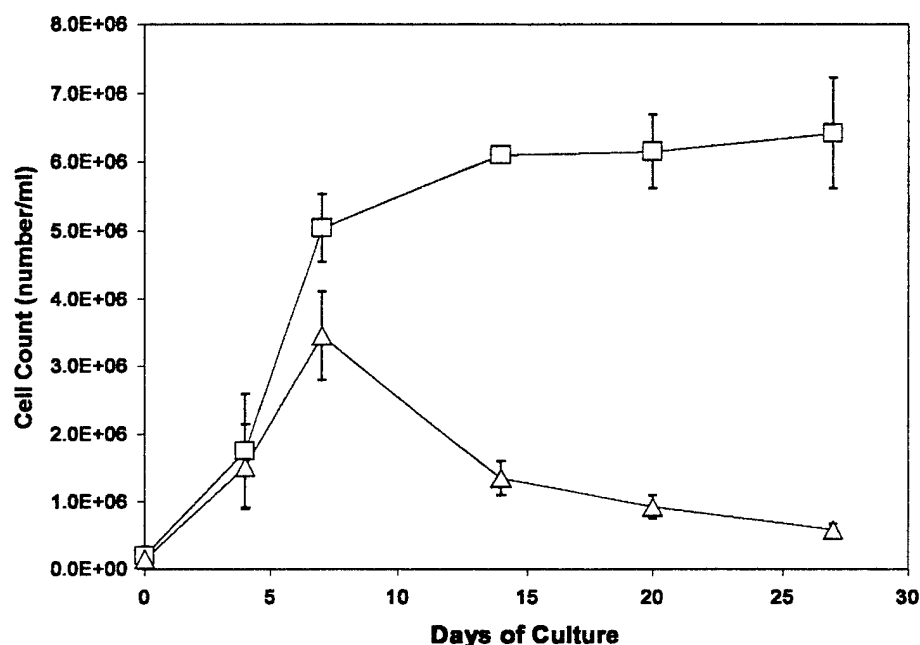


Figure 3. Hybridoma Cell Density During the Culture Period in the 2-L Gas Permeable Bags:
 (□) represents total cell numbers per milliliter
 (△) represents live cell numbers per milliliter

We also measured monoclonal antibody concentration in the serum-media during culture using an analytical Protein-A HPLC method.⁴ Figure 4 shows that antibody concentration in gas permeable bags increased rapidly during the first week of the culture. The concentration of antibody reached and maintained a maximum concentration of approximately 0.15 mg/mL after approximately 15 days. A total of approximately 11.3 g of mAb (before purification) was produced, using 40, 2-L gas permeable bags (approximately 280 mg mAb produced per bag).

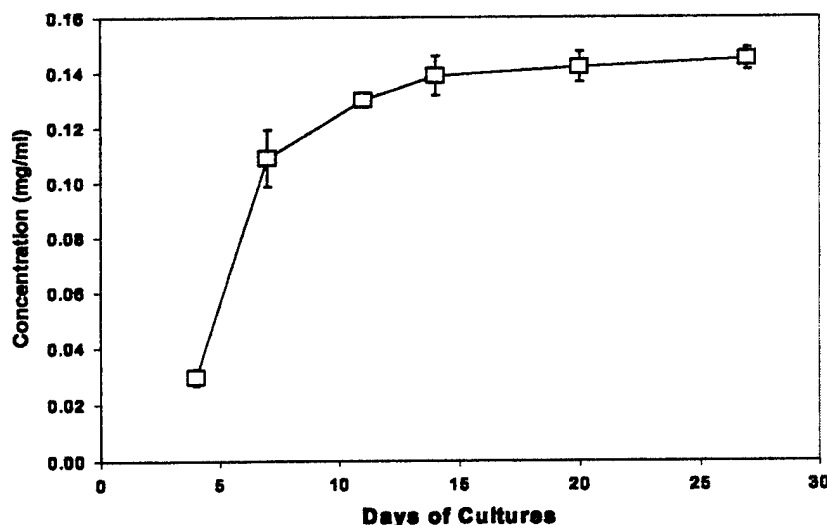


Figure 4. mAb Concentration in 2-L Gas Permeable Bags Measured by Analytical Protein-A Method

3.2 Purification of Antibodies.

Harvested antibody media from gas permeable bags were clarified using a centrifuge and pre-filtered by a disposable 0.2 μ m depth-filter. Monoclonal antibodies in culture media including serum were purified using Protein-A Sepharose column affinity chromatography. Harvested media were injected through the column and adsorbed antibodies were eluted with 50 mM citrate buffer, pH 3.0. Figure 5 shows the Protein-A chromatogram of antibody and pH elution data collected during purification. On this chromatographic profile, the mAb eluted sharply around 130 min with the pH change. The eluent from Protein-A chromatography was injected into a Sephadex G-25 desalting column for exchanging the buffer as a final formulation step with 20 mM Tris with 150 mM sodium chloride (TBS buffer) at pH 8.0, including 0.05% sodium azide as shown in Figure 6. The purified mAb product was sterile filtered with a 0.22- μ m filter.

An analysis of the in process samples collected during downstream processing was performed by the analytical Protein-A method as shown in Figure 7. The chromatograms clearly show that Protein-A is capable of providing total mAb purity higher than 95% in a single step from cell culture media.

Because purity and recovery are the critical parameters in downstream purification processes, it is important to keep track of values for both parameters in each step of the process. Representative purification data is given in Table 1. By calculation and comparison with the previous step, recovery for each step and total recovery were determined. Protein concentration and purity of the samples were measured by an analytical Protein-A method. Commercially obtained mouse IgG (Pierce) was used as a standard to calculate antibody concentrations. The overall recovery was 78%.

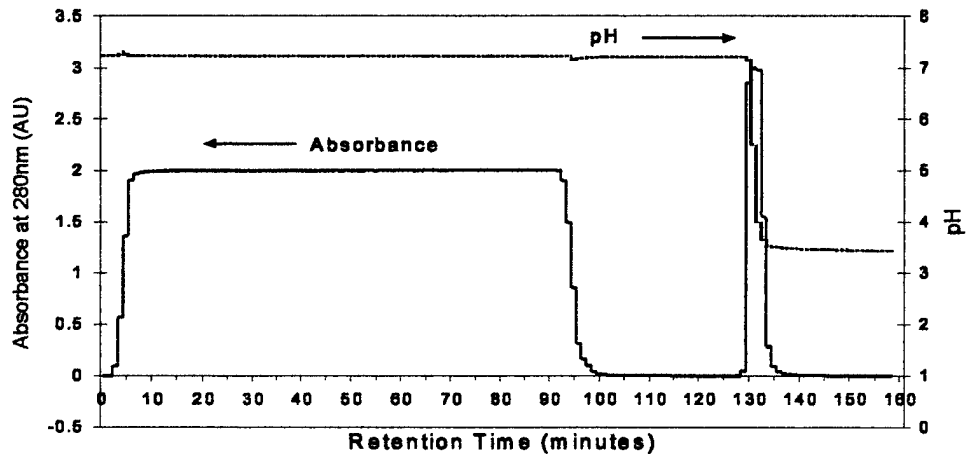


Figure 5. Preparative Scale Protein-A Chromatogram for Initial Purification of mAb from Crude Culture Media Harvested from Gas-Permeable Bags

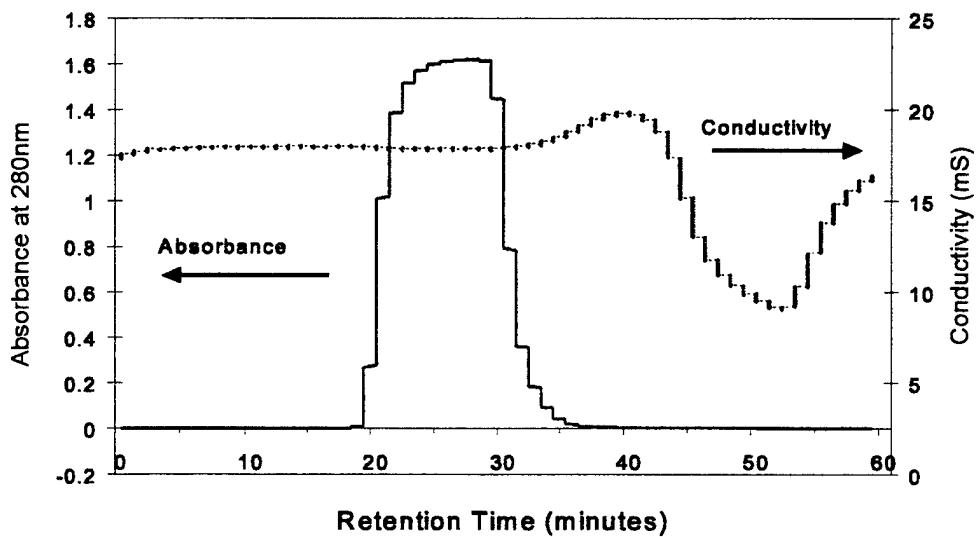


Figure 6. Preparative Scale of Desalting Column Chromatogram for Final Formulation of mAb from the Protein-A Column Eluent

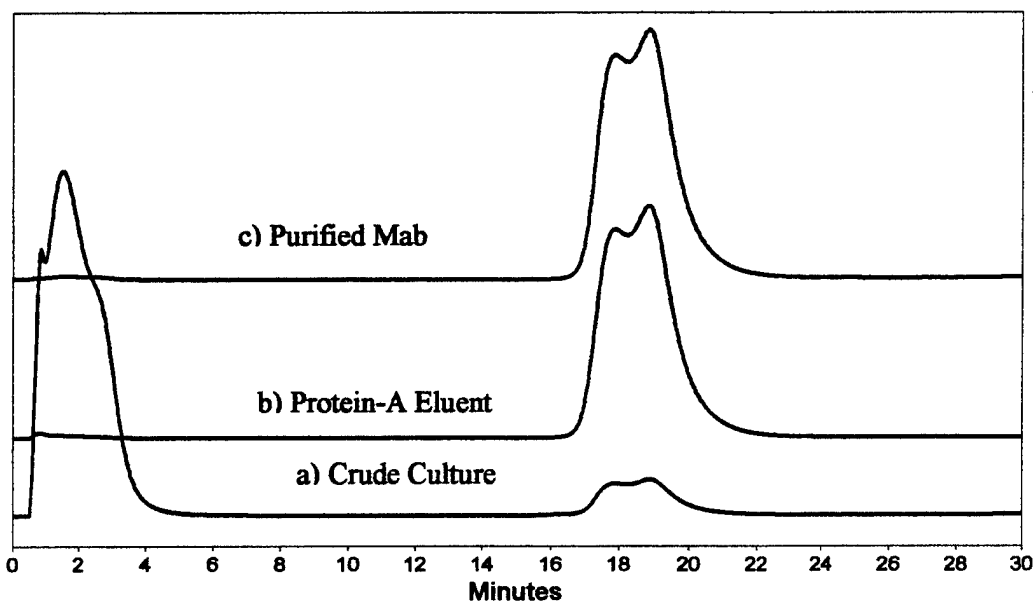


Figure 7. Chromatograms of the In-Process Samples Collected from a) Crude Cell Culture Media, b) Protein-A Eluent, and c) Desalting Column Pool. The samples were analyzed by an analytical Protein-A method.

Table 1. Purification Data for the Production of mAb: Lot No. E080301

| Step | Volume (mL) | Purity (%) | mAb Concentration (mg/mL) | Total mAb Quantity (mg) | Step Recovery (%) | Total Recovery (%) |
|--------------------------|-------------|------------|---------------------------|-------------------------|-------------------|--------------------|
| Cell Culture Supernatant | 8970 | 2.1 | 0.12 | 1076 | 100 | 100 |
| Protein-A Step | 216 | 96.2 | 4.00 | 864 | 80.3 | 80.3 |
| Desalting Step | 420 | 96.9 | 2.0 | 840 | 97.2 | 78.0 |

3.3 Product Analysis and Validation.

The purity of mAb is measured by an analytical GPC column (TosoHaas TSG-G3000SWXL) with a Beckman HPLC system and a SDS-PAGE method. Figure 8 shows the chromatograms obtained from the analytical GPC system. The purity of the mAb is 98.3%. The activity of the purified mAb is measured by the direct ELISA assay with a specific antigen. The assay works by comparing two antibodies as shown in Figure 8. The gold antibody was obtained from JPOBD as a reference standard. Figure 9 clearly shows that the purified mAb is active against the *F. tularensis*. More activity data are shown in the Appendix. Table 2 shows the summary data of the purified mAbs against the *Francisella tularensis* organism.

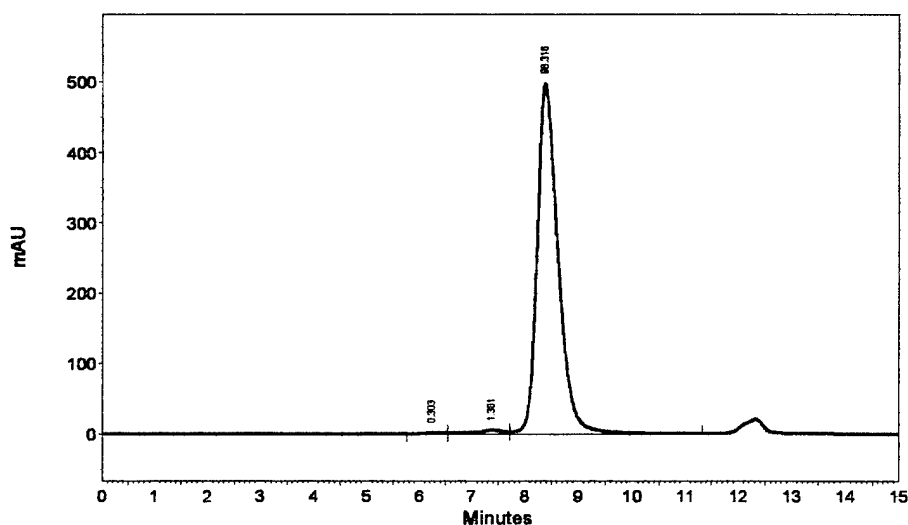


Figure 8. The Final Purified mAb Chromatogram Analyzed by an Analytical GPC System. The purity of mAb was measured by calculating percent peak area shown in the chromatogram.

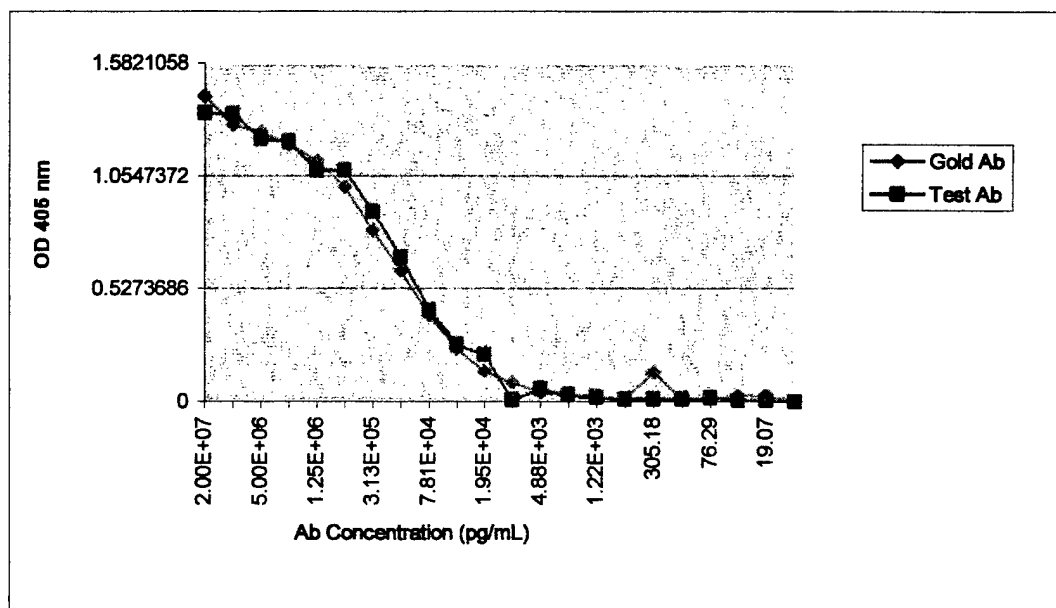


Figure 9. Direct ELISA Data (Lot No.: E080301) of the Purified mAb Produced by Gas Permeable Culture Bags and a Single Step Chromatography Purification Process

Table 2. Summary of the Purified mAb Lots

| Lot # | Total Mass (mg) | Concentration (mg/mL) | Purity (%) | Activity |
|--------------|------------------------|------------------------------|-------------------|-----------------|
| E071101 | - | - | 99.1 | active |
| E080201 | 435 | 4.58 | 96.9 | active |
| E080301 | 714 | 2.04 | 95.7 | active |
| E103101 | 623 | 2.15 | 96.9 | active |
| E111401 | 1058 | 2.10 | 96.7 | active |
| E010202 | 1221 | 3.10 | 97.4 | active |
| E010402 | 1340 | 3.40 | 97.1 | active |
| E010902 | 1058 | 2.70 | 94.3 | active |
| E011002 | 1128 | 3.10 | 94.3 | active |
| E011402 | 1058 | 2.10 | 97.1 | active |
| E011502 | 924 | 2.20 | 95.8 | active |

4. CONCLUSION

This report describes the biomanufacturing process development for the production of gram quantities of a monoclonal antibody. Two-liter gas permeable bags were used for growing the hybridoma cell lines for the mAb production. Our data showed that this *in vitro* culture system allows efficient production of mAbs. The supernatants harvested from the cultures were purified by a preparative affinity Protein-A chromatography. The simple small-scale biomanufacturing process produced 10 g of the purified mAb. The purified mAb was found to be active specific against *F. tularensis*.

The use of flexible, gas permeable bags is a reasonable alternative to the use of flask-style or hollow fiber bioreactors for monoclonal antibody production. Gas permeable bags were seeded easily using a syringe barrel and a peristaltic pump. Materials required for gas permeable bag culture are commercially available, as a complete kit. The gas permeable property of the bags allowed the use of standard laboratory incubators (5% CO₂ v/v and 37 °C). In addition, as the bags are essentially a closed system after inoculation, the risk of bacterial contamination was significantly reduced. In this study, no antibiotics were used in the growth media and contamination was rare and sampled frequently. In addition to the technical consideration, the gas permeable bags provided significant economic advantage.

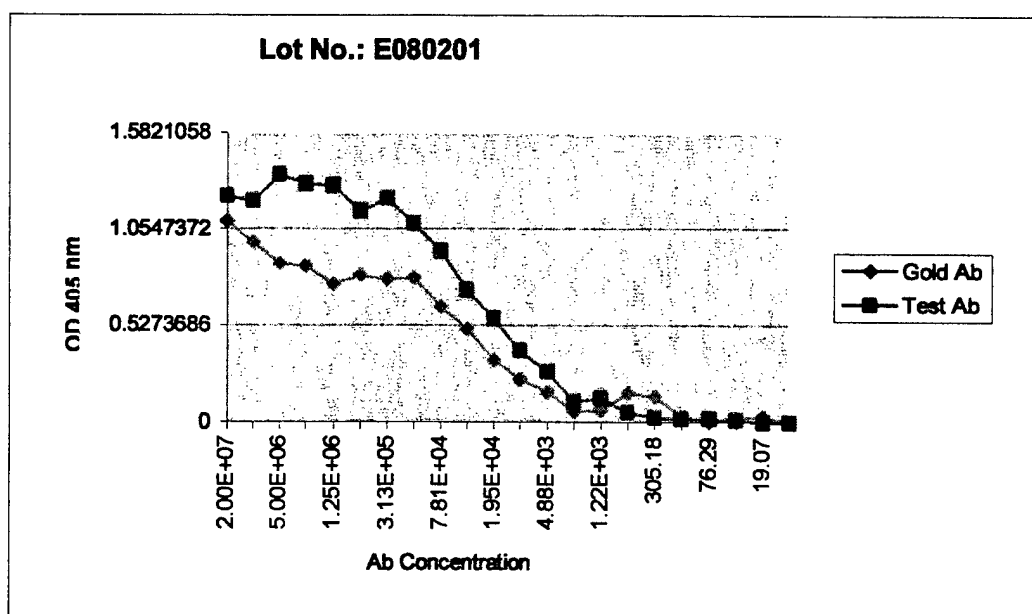
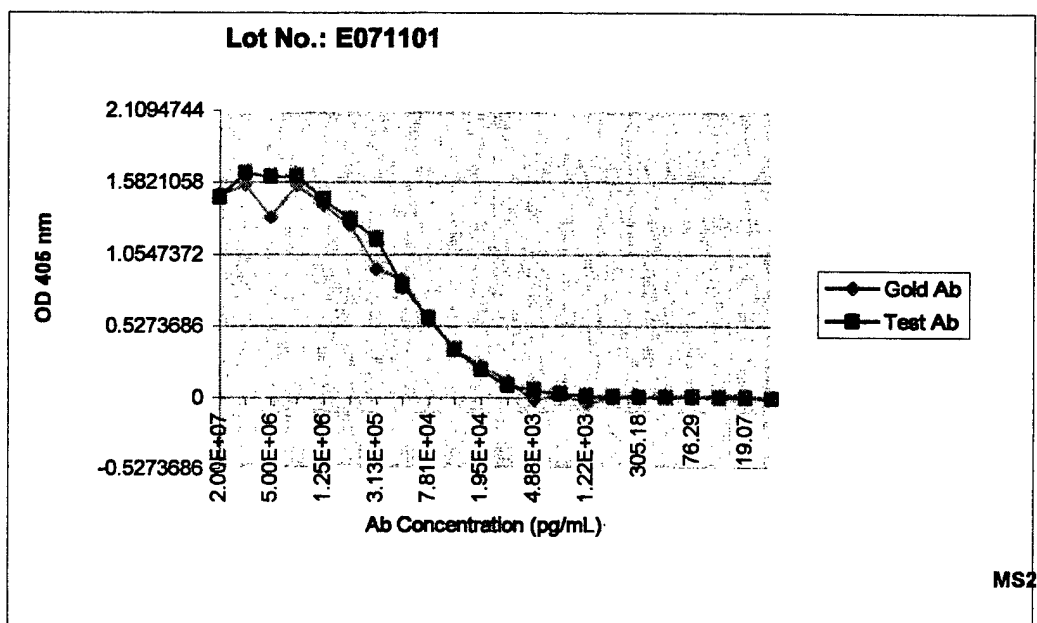
*Park, J. T.; Cork, S.; Coliano, T.; Cao, C.; Menking, D.; O'Connell, K.; and Valdes, J. J.; Research and Technology Directorate, U.S. Army Edgewood Chemical Biological Center, July 2002, unpublished data.

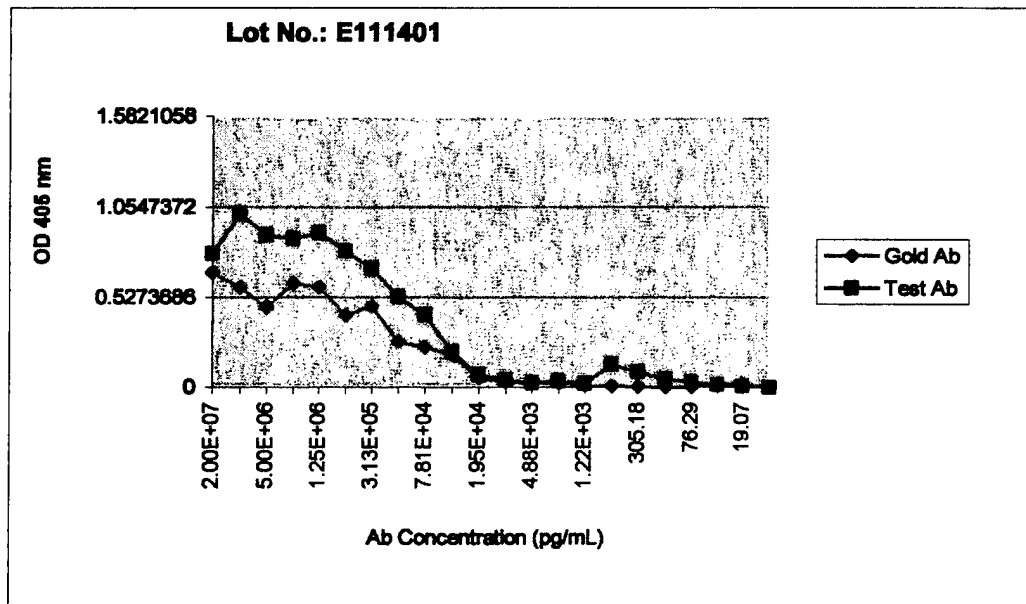
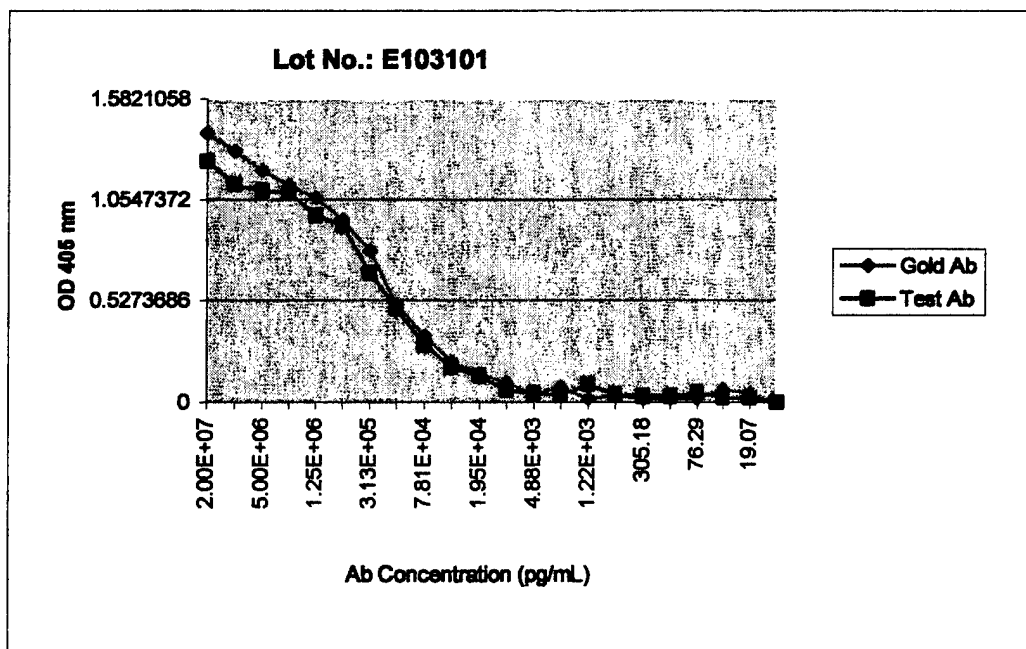
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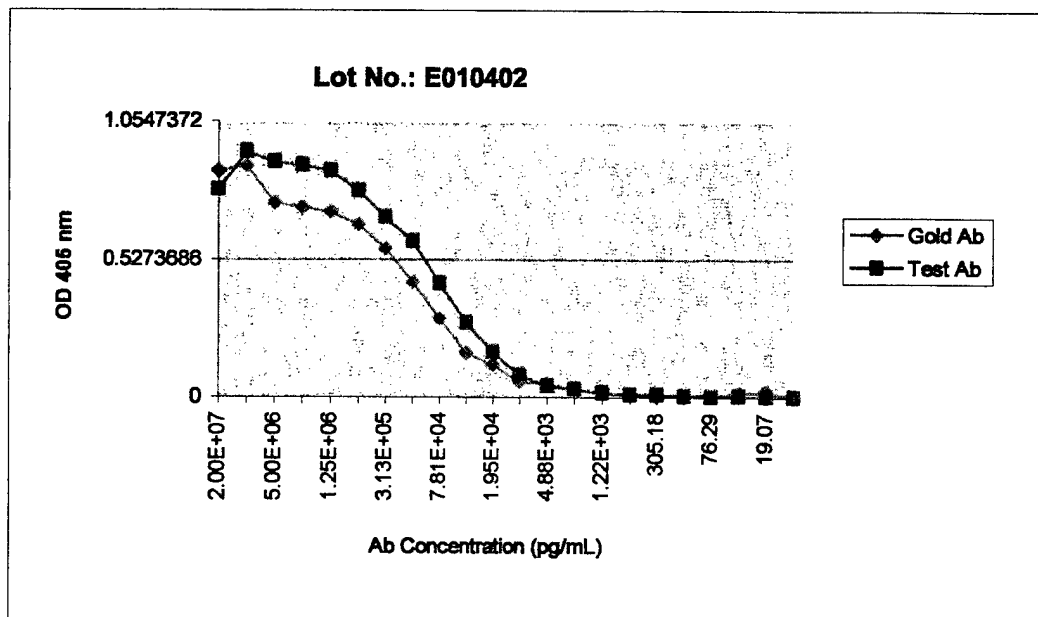
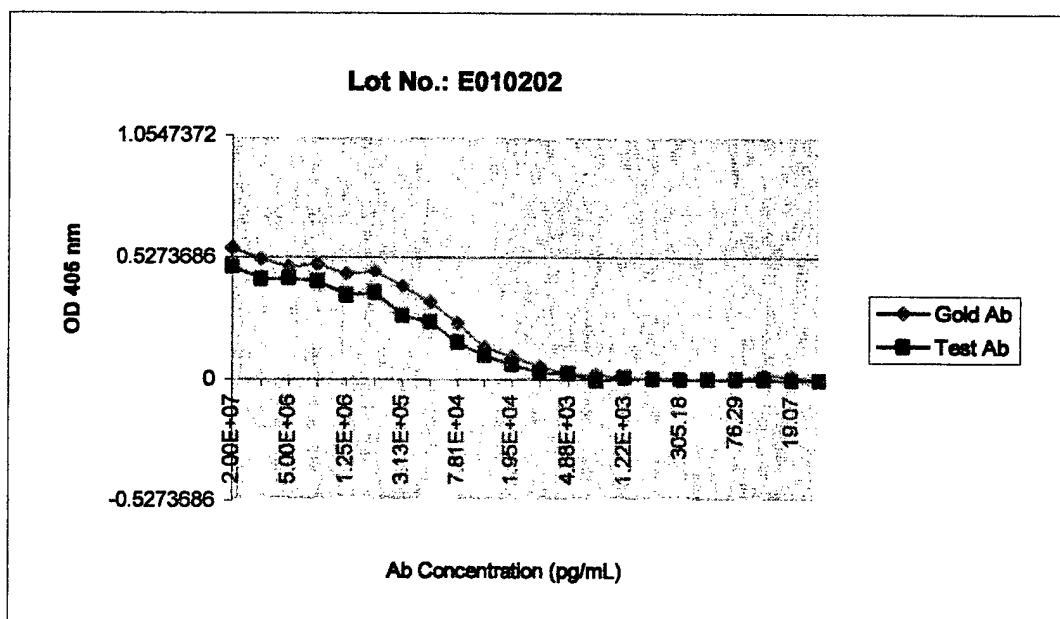
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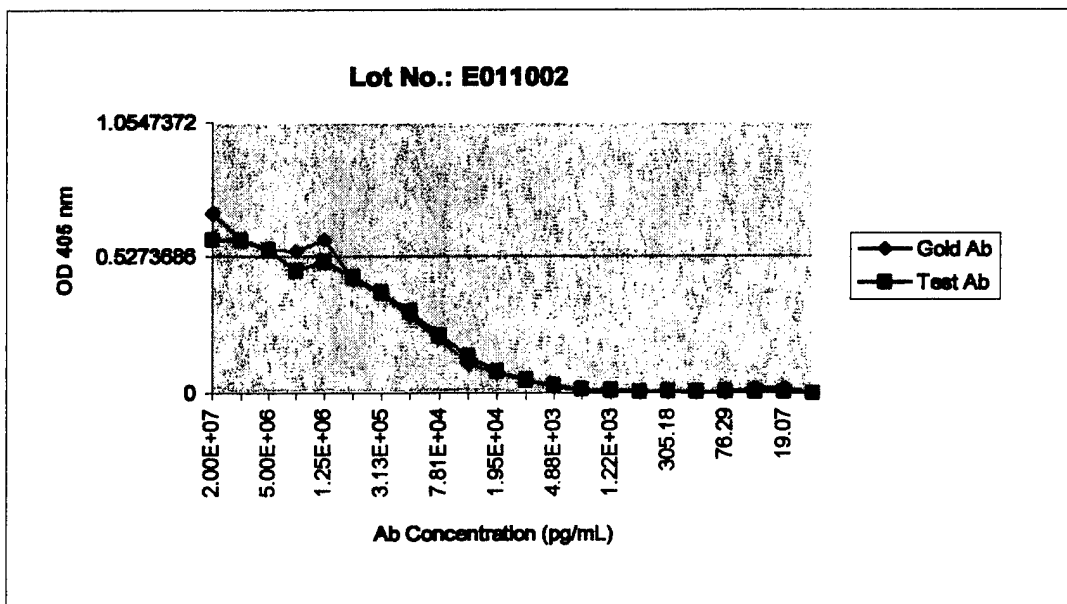
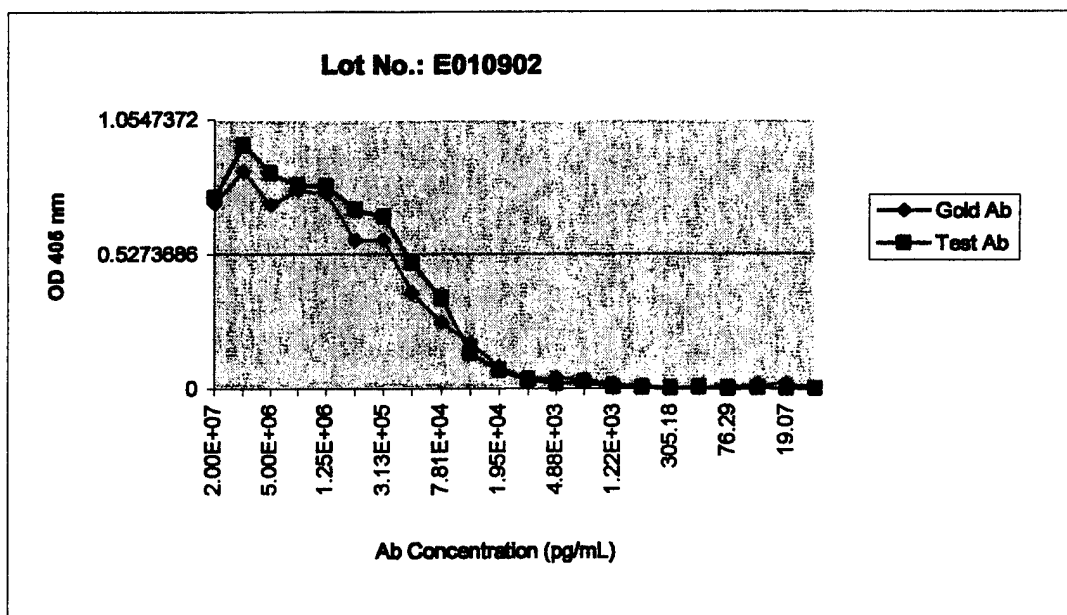
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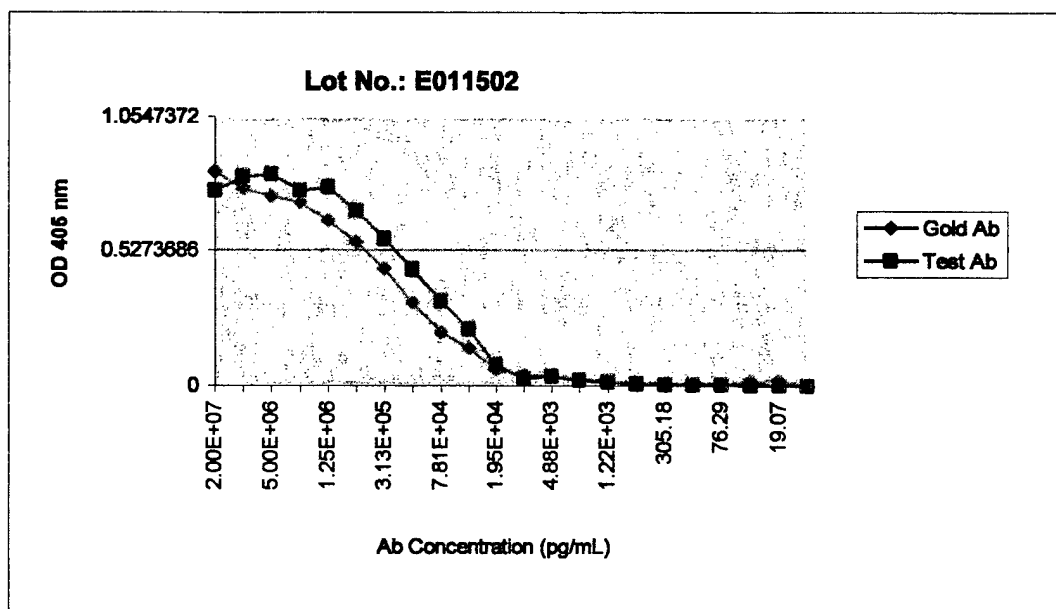
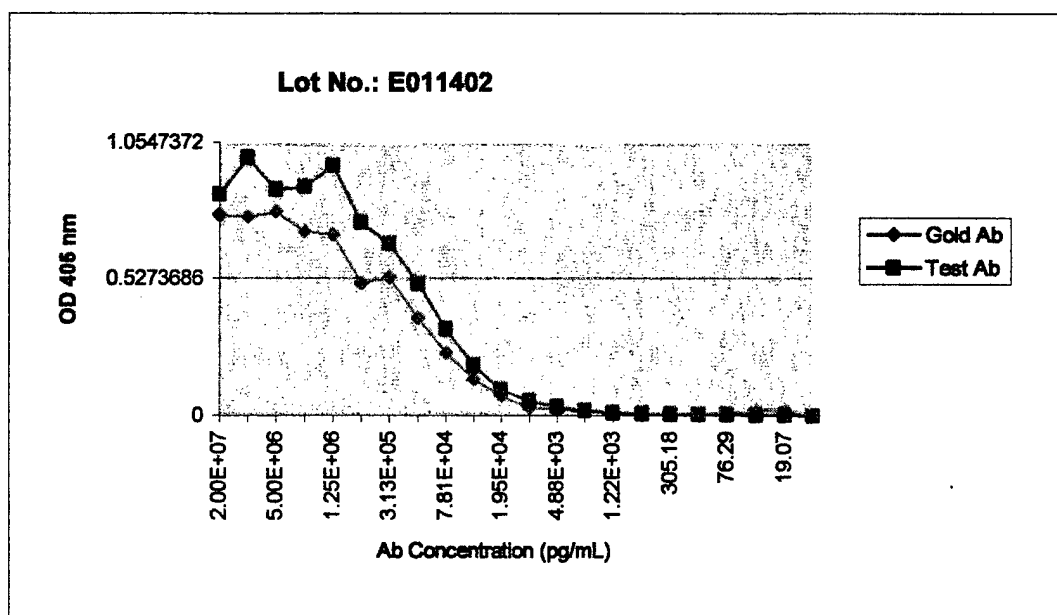
APPENDIX ELISA DATA OF mAb BATCH LOTS











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